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Meta-topolin Enhanced *In Vitro* Regeneration, Acclimatization, and Genetic Stability Assessment of Regenerated Watermelon (*Citrullus lanatus* Thunb.)

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ABSTRACT

The production of seedless watermelons, primarily through triploid varieties, has surged to meet the growing consumer demand, especially due to the convenience of eating. However, triploid watermelon production is time-consuming, and seed production is tedious. Hence, *in vitro* propagation has become an alternative, but it faces challenges such as low regeneration response, poor rooting, and low *ex vitro* establishment. These issues can be addressed by applying aromatic cytokinin *meta-topolin* (*m*T) in the regeneration system. Hence, this study aims to investigate the efficacy of *meta-topolin* (*m*T) compared to 6-benzyl adenine (BA) for in vitro regeneration and acclimatization of *Citrullus lanatus* (Thunb.). The effects of aromatic cytokinins BA and *m*T (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) on multiple shoot production from cotyledonary node explants of watermelon were evaluated. The highest shoot production (25.24 shoots/explant) was observed with 1.5 mg/L *m*T, while BA (1.0 mg/L) produced 11.36 shoots/explant. Rooting response in MS medium with indole-3-butyric acid (IBA 0.5 to 2.5 mg/L) showed the best results in IBA 1.0 mg/L with *m*T-derived shoots (1.5 mg/L), producing 13.33 roots per shoot, compared to 5.62 roots per shoot from BA-derived shoots (1.0 mg/L). After four weeks of acclimatization, *m*T-derived plants had a 97% survival, while BA-derived plants had 84%. Additionally, *m*T-derived plants had significantly higher photosynthetic pigments (chlorophyll a (9.2%), b (11.3%), and carotenoids (29.1%)). RAPD and SCoT markers confirmed the genetic

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stability of the regenerated plants. This research will aid in developing high-quality planting material to produce commercial triploid watermelon plants.

Keywords: Acclimatization, chlorophyll, *meta*-topolin, molecular markers, rhizogenesis, shoot induction

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a widely cultivated fruit crop of significant economic and nutritional importance worldwide. Watermelon is an excellent functional food because it provides vital sources of essential nutrients, like vitamins, minerals, and antioxidants (Athanasiadis et al., 2023; Manivannan et al., 2020). The popularity of seedless watermelons has increased because of their convenience. Seedless watermelon is a hybrid of two inbreds, where the male parent is diploid, and the female parent is a tetraploid (Kaseb et al., 2023; Subasinghe Arachchige et al., 2022). However, the production of triploid watermelon is challenging and time-consuming compared to diploid cultivars (Phat et al., 2015; Solmaz et al., 2018). Hence, *in vitro* propagation, which can produce a large number of clones in a short duration, is an alternative solution to this issue. Although *in vitro* propagation in watermelon has been carried out earlier, several limitations prevent extensive use of this method. Previously, *in vitro* regeneration studies using triploid watermelon (var. Arka manik) gave low frequency of shoot production (Nasr et al., 2004; Thomas et al., 2000), poor rooting and low *ex vitro* establishment as the limiting factors (Shalaby et al., 2008; Vinoth & Ravindhran, 2016).

Several factors impact the establishment of *in vitro* regeneration in watermelon, including genotype, explant type, culture conditions, and hormonal treatments (Ameri et al., 2015; Vinoth & Ravindhran, 2016). Growth regulators play a significant role in the *in vitro* regeneration of watermelon, particularly cytokinins (Badr-Elden et al., 2012; Gnamien et al., 2013). However, different varieties or cultivars of watermelon react differently to the type and concentration of cytokinin; such different responses were observed among watermelon cultivars such as Arka Manik, Giza 1 and Giza 21 (Vinoth & Ravindhran, 2016; Zakaria et al., 2007). Thus far, the optimal shoot proliferation from watermelon explants was noted in a BA-supplemented medium. According to previous reports, the disadvantages of the application of 6-benzyladenine (BA) in plant multiplication through the *in vitro* method involve the production of unhealthy shoots, decreased root formation, and poor survival during acclimatization rate (Ameri et al., 2015; Badr-Elden et al., 2012; Vinoth & Ravindhran, 2016). These physiological disorders have the potential to negatively affect the quality of *in vitro* plant production. Hence, replacing BA with *m*T is an upcoming trend (Nowakowska & Pacholczak, 2019; Werbrouck et al., 2008) and is recommended for improving the regeneration frequency of watermelon.

The meta-topolin (*m*T) is a hydroxylated form of 6-benzylaminopurine and an innate aromatic cytokinin that was originally extracted from Poplar plant leaves (Strnad et al., 1997; Werbrouck et al., 1996). Topolins are currently used as substitutes for cytokinin, which is most potent in plant micropropagation. It has been observed that topolins increase shoot proliferation, maintain hormonal stability, and improve rooting efficiency in bananas (Aremu et al., 2012; Bairu et al., 2008). Using *meta*-topolin instead of BA usually produces a higher number of quality shoots with increased chlorophyll content and better rooting with an improved acclimatization rate (Koszeghi et al., 2014; Nowakowska et al., 2019). As far as we are aware, no study has been done on evaluating the impact of *m*T on *in vitro* propagation of watermelon (Arka manik), covering aspects from *in vitro* shoot induction, rooting and acclimatization. However, it is also necessary to ensure that side effects such as the somaclonal variation are not prevalent due to the use of *m*T.

Clonal propagation with increased cytokinin concentration resulted in somaclonal variations in strawberries and bananas (Biswas et al., 2009; Bidabadi & Jain, 2020). Changes in the plant genome during the regeneration of plants due to somaclonal variation may be hereditary or epigenetic (Duta-Cornescu et al., 2023). One of the contributing factors for somaclonal changes and mutations in the regenerated plantlets is the use of synthetic plant growth regulators, among others, such as the alterations in the nutrients supplied in the media, prolonged subculturing interval of plantlets in tissue culturing systems, and altered pH of the media (Duta-Cornescu et al., 2023). Since this study attempts to use *m*T for *the in vitro* production of watermelon, testing the *in vitro* clonally grown plants for genetic homogeneity is of utmost significance in producing tissue culture plants of elite genotype. It has long been advised to use molecular markers to examine the genetic homogeneity of *vitro*-cloned plants (Al-Khayri et al., 2022; Chirumamilla et al., 2021; Gantait et al., 2022; Koh et al., 2024). Molecular markers such as random amplified polymorphic DNA (Deoxyribonucleic acid; RAPD) and the start codon targeted (SCoT) polymorphism (Ajithan et al., 2020; Bisht et al., 2024; Elayaraja et al., 2019; Joshi et al., 2023; Sathish et al., 2022; Vasudevan et al., 2017) can be utilized as reliable tools to assess the genetic variations in regenerated plants. For establishing genetic fidelity via molecular marker-assisted analysis, start codon targeted (SCoT) polymorphism marker systems have been used as simple, reliable and highly reproducible tools (Bhattacharyya et al., 2023; Rai, 2023). Thus, two types of molecular markers were used in this study to screen the genetic stability of the *in vitro*-raised watermelon plantlets.

Hence, our current study aims to investigate the efficiency of *m*T in comparison with BA on *in vitro* shoot multiplication, rooting, and acclimatization and confirm the genetic homogeneity of plants using RAPD and SCoT molecular markers in regenerated watermelon.

MATERIALS AND METHODS

Explant Preparation and Media

Murashige and Skoog medium (Murashige & Skoog, 1962) containing 100 mg/L of myo inositol, and 3% sucrose was used as the culture medium for the present study. The pH of the medium was adjusted to 5.6 by using 1 N HCl and 1 N NaOH before adding 0.8% agar, and the medium was autoclaved for 15 min at 121°C. All the plant growth regulators were introduced into the media prior to autoclaving. All the required chemicals were purchased from HiMedia®, Mumbai, India. All the cultures were maintained at 25 ± 2°C under a 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1}.

The triploid watermelon seeds used in this study, namely Arka manik, were provided by the Indian Institute of Horticultural Research (IIHR) in Bengaluru, India. Surface sterilization of mature seeds was carried out using mercuric chloride (0.1%) for 3 minutes, after which the seeds were washed three times with distilled water. The sterilized seeds were inoculated into MS basal media (Murashige & Skoog, 1962) supplemented with agar (0.8% W/V) and 3% sucrose and kept in complete darkness for three days followed by in light (40 µmol m^{-2} s^{-1}) at 25±2°C for four days under a 16/8 h photoperiod (Figure 1a). Watermelon cotyledonary node explants (10–15 mm, Figure 1b) were prepared by removing cotyledons, apical meristems, and persistent hypocotyls from 7-day-old *in vitro*-grown plants.

Effect of Aromatic Cytokinins

Cotyledonary node explants were excised from 7-day-old *in vitro* seedlings and inoculated on MS medium (Murashige & Skoog, 1962) supplemented with various plant growth regulators. The medium for *in vitro* regeneration was standardized using various BA and *m*T concentrations. For multiple shoot production, MS basal medium supplemented with BA and *meta*-topolin (*m*T) at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) was used individually. As a control, cytokinin-free basal MS media was employed. All cultures were transferred to a fresh medium every two weeks of incubation. After six weeks, the mean number of multiple shoots per explant, mean shoot length, and percentage of explants' response were recorded. The cultures were maintained at 25±2°C with a 16/8 hr photoperiod and 40 µmol m^{-2} s^{-1} light intensity.

Optimizing the BA and *m*T-Derived Shoots for Root Induction

BA- or *m*T-derived, healthy and well-elongated shoots (≥4 cm) with more than four fully expanded leaves were isolated separately in two groups (BA- and *m*T-derived shoots) from six-week-old cultures. After that, the shoots were transferred onto MS medium augmented with different doses of Indole-3-butyric acid (IBA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) to

evaluate the efficiency of the rooting response. A basal medium devoid of auxins was assigned as a control. After six weeks of culture, the percentage of explant response, the mean number of roots and root length were measured. The cultures were maintained at 25±2°C with a 16/8 h photoperiod and 40 µmol m^{-2} s^{-1} light intensity.

Acclimatization of Regenerated Plants

The rooted watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were removed from the rooting media. The plants were thoroughly cleaned under running tap water to get rid of the gel residue that adhered to the roots. The rooted plantlets derived from BA and *m*T treatments were acclimatized in paper cups containing a pot mixture of sand and soil (1:1 v/v/v ratio). The acclimatized plants were covered with polyethylene bags for two weeks, and then the plantlets were transferred to the greenhouse condition. After four weeks of acclimatization, the percentage of plants that survived, the number of leaves per plant and shoot length were recorded.

Quantification of Photosynthetic Pigments

The leaves' from acclimatized watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were collected for the quantification of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) at five different intervals (1, 7, 14, 21 and 28 days). Fresh leaves were harvested from 5 plants derived from *m*T (1.5 mg/L) treatments, approximately 100 mg from each plantlet was subjected to chlorophyll pigment extraction, and the experiments were carried out with three replicates. Similarly, fresh leaves were harvested from 5 plants derived from BA (1.0 mg/L) treatments, and approximately 100 mg from each plantlet was subjected to chlorophyll estimation. The analysis was carried out with methanolic extracts of leaves using a colorimetric method by Lichtenthaler (1987), with minor modifications as detailed by Aremu et al. (2012) and Vasudevan et al. (2017). The pigment concentrations were expressed as µg per gram of fresh weight.

Plant Total Genomic DNA Extraction and Genetic Fidelity Analysis

The acclimatized watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were examined for genetic fidelity analysis. A genomic DNA extraction kit (Sigma–Aldrich, USA) was used for genomic DNA extraction from 10 plantlets (5 plants derived from *m*T (1.5 mg/L) and 5 plants derived from BA (1.0 mg/L) treatments, these plantlets were selected from a total of 50 plantlets for each treatment. The quantification of the genomic DNA was performed by using a NanoDrop spectrophotometer (Biodrop, UK). Analysis of clonal fidelity was performed using 10 RAPD markers and 15 SCoT markers.

A total of 10 RAPD primers (Table 4) and 15 SCoT primers (Table 5) were used for DNA amplification to evaluate the clonal fidelity of *in vitro* regenerated plants. Polymerase

Chain Reaction (PCR) amplification for RAPD was carried out according to the procedure followed by Vasudevan et al. (2017). PCR amplification was carried out by using Taq 2X master mix RED containing Tris HCl (pH 8.5), $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween® 20, 0.4 mM of each dNTP, 0.2 units/µl of Taq DNA polymerase (AMPLIQON, Denmark), template DNA (100 ng) and 5 pmol of specific primers. A reaction mixture containing 25 µl of PCR mixture was prepared by adding 12.5 µl of 2X Taq PCR master mix, 7.5 µl of nuclease-free water, 2 µl of the required primer and 3 µl of a specific DNA sample. PCR was carried out in a PTC-100® thermal cycler (MJ Research Inc., USA) programmed with initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, annealing at 50°C (37°C for RAPD) for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR-amplified products were separated by electrophoresis in a 1.2% agarose gel and documented by a gel documentation system (UVITEC, France). For PCR-based marker studies, PCR was repeated at least three times with each primer, and only repetitive and scorable bands were used for genetic analyses of regenerated plants (Agarwal et al., 2015).

Statistical Analysis

All the experiments were replicated three times (except acclimatization, which had five replications). Each treatment consisted of 20 explants. The data were examined using IBM Statistical Package for the Social Sciences (SPSS) Statistics version 25. Mean values were compared using Duncan's multiple range test (DMRT) and one-way analysis of variance (ANOVA) at the 5% significance level. The results were expressed as the mean ± standard error (SE).

RESULTS AND DISCUSSION

Production of Multiple Shoots

Cotyledonary node explants derived from 7-day-old *in vitro*-grown seedlings (Figures 1a and b) were inoculated on MS medium containing two different cytokinins (*m*T and BA) individually at various concentrations ranging from (0.5–2.5 mg/L). After two weeks of incubation, multiple shoots were initiated from the cotyledonary node explants in both BA and *m*T treatments (Figures 1c and d). Among them, the media fortified with *m*T exhibited better results, irrespective of the concentration, than media supplemented with BA. The maximum number (25.24) of shoots were recorded in MS medium supplemented with 1.5 mg/L *m*T (Figure 1e, Table 1), followed by 2.0 mg/L *m*T (19.16) and 1 mg/L *m*T (13.06). In treatments with BA, the highest number of shoots was obtained in 1 mg/L BA (11.36 shoots per explant), followed by 1.5 mg/L BA (9.00 shoots) and 0.5 mg/L BA (8.00 shoots), the highest being only 50% compared with *m*T at 1.5 mg/L.

Table 1
Effect of mT and BA on shoot induction from cotyledonary node explants of watermelon in MS media after 6 weeks of culture

Cytokinins	Concentration (mg/L)	Percentage of response	No. of shoots per explant	Shoot length (cm)
Control	0.0	0.0	0.0	0.0
BA	0.5	35.33 ± 0.05 ^{h}	8.00 ± 0.32 ^{ef}	2.05 ± 0.03 ^{b}
	1.0	75.00 ± 1.45 ^{b}	11.36 ± 0.14 ^{cd}	2.45 ± 0.06 ^{b}
	1.5	57.66 ± 1.02 ^{e}	9.00 ± 0.80 ^{e}	2.21 ± 0.02 ^{b}
	2.0	45.00 ± 1.45 ^{g}	7.81 ± 0.12 ^{fg}	2.62 ± 0.04 ^{b}
	2.5	66.33 ± 1.45 ^{cd}	5.54 ± 0.64 ^{h}	2.54 ± 0.03 ^{b}
*m*T	0.5	42.66 ± 1.25 ^{g}	7.38 ± 0.14 ^{fg}	4.18 ± 0.04 ^{a}
	1.0	51.00 ± 1.16 ^{f}	13.06 ± 0.56 ^{c}	4.43 ± 0.06 ^{a}
	1.5	84.33 ± 0.37 ^{a}	25.24 ± 0.80 ^{a}	4.80 ± 0.03 ^{a}
	2.0	72.00 ± 1.02 ^{b}	19.16 ± 0.34 ^{b}	4.56 ± 0.04 ^{a}
	2.5	61.66 ± 1.15 ^{cd}	12.84 ± 0.12 ^{c}	4.12 ± 0.02 ^{a}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine

Table 2
Effect of auxin (IBA) on root induction from cytokinin-derived shoots of watermelon in MS medium after 6 weeks of culture

Cytokinin derived-shoots	IBA (mg/L)	Percentage of response	Number of roots per shoots	Length of roots (cm)
BA derived	0.5	54.66 ± 0.88 ^{f}	2.64 ± 0.07 ^{g}	1.53 ± 0.06 ^{c}
shoots	1.0	75.66 ± 1.33 ^{c}	5.62 ± 0.08 ^{e}	2.04 ± 0.02 ^{b}
	1.5	71.00 ± 1.20 ^{d}	3.46 ± 0.11 ^{f}	1.74 ± 0.06 ^{c}
	2.0	65.00 ± 1.15 ^{e}	3.41 ± 0.06 ^{f}	1.35 ± 0.04 ^{c}
	2.5	54.00 ± 1.00 ^{f}	1.49 ± 0.29 ^{h}	1.71 ± 0.03 ^{c}
*m*T derived	0.5	78.33 ± 1.33 ^{c}	8.28 ± 0.13 ^{c}	1.17 ± 0.03 ^{c}
shoots	1.0	96.66 ± 1.20 ^{a}	13.33 ± 0.27 ^{a}	3.61 ± 0.04 ^{a}
	1.5	87.66 ± 1.20 ^{b}	9.54 ± 0.08 ^{b}	2.23 ± 0.08 ^{b}
	2.0	76.00 ± 0.57 ^{c}	7.20 ± 0.16 ^{d}	2.80 ± 0.02 ^{b}
	2.5	64.66 ± 1.85 ^{e}	5.12 ± 0.24 ^{e}	2.47 ± 0.08 ^{b}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine; IBA = Indole-3-butyric acid

In the shoot multiplication stage, it was found that with an increase in the concentration of *m*T up to 1.5 mg/L, there was a gradual increase in the number of shoots (from 7 to 25 shoots). Our study showed that with an increase in concentration from 1.5 to 2.5 mg/L, a gradual decline in shoot number was observed, specifically, 12.84 shoot/explant in

comparison to 1.5 mg/L (25.24 shoots). Profuse growth and development of shoots were noticed in optimal doses of *m*T-fortified medium (Figure 1f). Likewise, in BA treatments, the number of shoots increased with an increase in BA concentration up to 1 mg/L, but thereafter, a gradual decrease in the shot number was recorded. Similar results were observed by Badr-Elden et al. (2012), whereby the use of a higher concentration of BA also showed reduced growth and development in watermelon *in vitro* regeneration. It appears that *m*T at 1.5 mg/L was the best for enhancing shoot production from cotyledonary node explants of watermelon. At the same time, *m*T applied at the same concentration was the most beneficial for shoot elongation (Figure 1g, Table 2).

As an alternative to commonly used cytokinins (BA), *m*T offers great potential for enhancing *vitro* regeneration and shows beneficial traits such as improved shoot production and increased shoot length in several crops (Gantait & Mitra, 2021). From the mechanism perspective, the presence of a hydroxy (-OH) group in the side chain of topolins over other cytokinins makes the absorption of *m*T easier, which allows the accumulation of O-glycosides (Krishna et al., 2021; Lalthafamkimi et al., 2021). These O-glucoside conjugates can convert into active free bases and supply cytokinins to the plants for a prolonged period, which leads to normal growth and development of plant cells and tissues (Erisen et al., 2020; Gentile et al., 2017). In the present study, a greater number of multiple shoots were produced in 1.5 mg/L *m*T-supplemented medium. These results clearly showed the use of *m*T as a potential alternative for BA in micropropagation of watermelon. In the case of watermelon, no such report has been available on the efficacy of *m*T in shoot proliferation.

Induction of Roots from *m*T- and BA-Derived Shoots

The *in vitro*-regenerated shoots (obtained from 1.5 mg/L *m*T and 1.0 mg/L BA) were inoculated in different concentrations of IBA (0.5 – 2.5 mg/L) individually, supplemented in MS medium along with control for assessing the efficiency of auxins on rooting traits. Among the treatments tested, the initial root induction was noticed from the *m*T-derived shoots after one week of incubation in a 1 mg/L IBA-supplemented medium (Figure 1h). After 6 weeks of culture, the maximum number (13.33) of roots was found to be induced in 1 mg/L IBA-supplemented medium.

In the case of BA-derived shoots, the highest number of roots (5.62 ± 0.08) and root length (2.04 ± 0.02) per shoot were recorded in 75% of cultures on MS medium augmented with 1 mg/L IBA. The efficiency of *in vitro* rooting of *m*T-derived shoots was high as compared to BA (Table 2). Similar to our studies, Ahmad and Anis (2019) observed that mT (2 mg/L) derived shoots were found to be more effective than BA-derived shoots for rhizogenesis in *Pterocarpus marsupium* (Roxb.) using cotyledonary node explants.

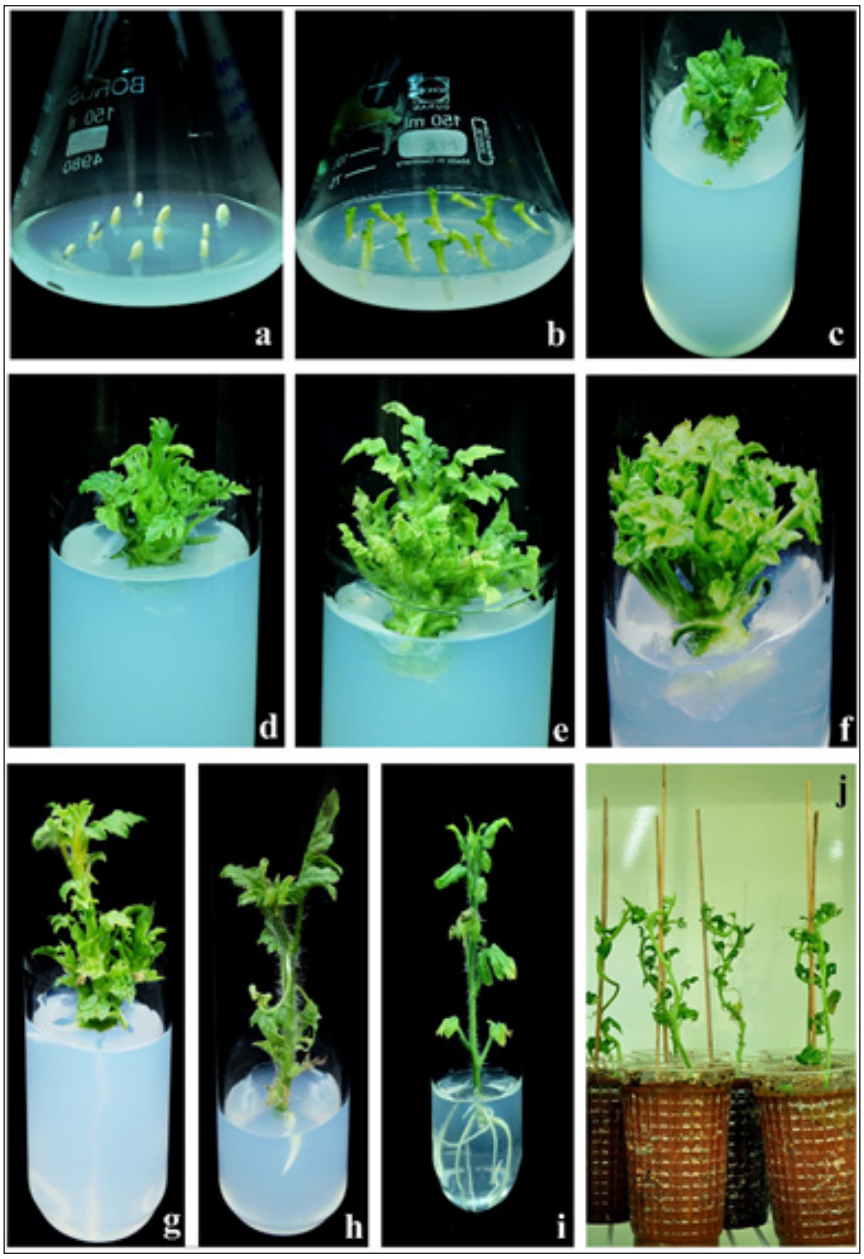

Figure 1. The effect of *m*T on the multiple shoot's induction and rooting efficiency of watermelon cv. Arka manik. (a) Seeds in MS medium, (b) Cotyledonary node explants, (c) Initiation of shoot buds in medium with BA (1 mg/L), after 2 weeks of initial culture, (d) Multiple shoot induction in medium with *m*T (1.5 mg/L), after 2 weeks, (e) Proliferation of shoot on MS medium containing *m*T (1.5 mg/L) after 4 weeks of culture, (f) shoot multiplication on medium containing *m*T (1.5 mg/L) after 6 weeks, (g) Elongated shoots on medium with *m*T (1.5 mg/L) after 6 weeks, (h) Elongated shoot on medium with IBA (1 mg/L) for rooting, (i) Shoots with profuse rooting on medium with IBA (1 mg/L), after 6 weeks of culture, (j) acclimatized plants

Acclimatization of BA- and *m*T-Derived plantlets

The *in vitro*-regenerated plantlets (obtained from 1.5 mg/L *m*T followed by 1 mg/L IBA-supplemented medium) were successfully acclimatized under *ex vitro* growing conditions for 4 weeks (Figure 1j). A more than 97% survival rate was recorded, confirming the effective root growth and development of acclimatized watermelon plantlets. The rooting efficiency of *m*T-derived plantlets was better, with a good survival rate of regenerated plantlets (97%) in *ex-vitro* conditions, compared to an 84% survival rate with BA-regenerated plantlets (Table 3). Some reports have documented that the type and dose of cytokinins have a profound effect on *ex vitro* rooting and acclimatization (Bairu et al., 2007; Hlophe et al., 2020). We have observed a moderate acclimatization success of BA-derived plantlets with a survival rate of 80% to 84%. Similarly, the negative effect of BA on root formation has been reported, resulting in deprived acclimatization rates in many plant species (Bairu et al., 2007; Werbrouck et al., 1996).

Table 3
Survival percentage of mT and BA-derived plants after 4 weeks of acclimatization

Cytokinin derived-shoots	IBA (mg/L)	Percentage of plantlets survived	Number of leaves per plantlet	Shoot length (cm)
BA derived shoots	0.5	81.66 ± 0.88 ^{d}	9.64 ± 0.07 ^{e}	9.53 ± 0.06 ^{e}
	1.0	84.00 ± 1.33 ^{c}	12.62 ± 0.08 ^{c}	10.04 ± 0.02 ^{d}
	1.5	82.33 ± 1.20 ^{d}	10.46 ± 0.11 ^{d}	9.74 ± 0.06 ^{e}
	2.0	81.66 ± 1.15 ^{d}	9.41 ± 0.06 ^{e}	9.35 ± 0.04 ^{e}
	2.5	80.00 ± 1.00 ^{d}	9.49 ± 0.29 ^{e}	8.71 ± 0.03 ^{f}
*m*T derived shoots	0.5	88.33 ± 1.33 ^{b}	12.28 ± 0.13 ^{c}	11.17 ± 0.03 ^{c}
	1.0	97.60 ± 0.20 ^{a}	15.33 ± 0.08 ^{a}	13.61 ± 0.08 ^{a}
	1.5	89.66 ± 0.34 ^{b}	13.64 ± 0.27 ^{b}	12.83 ± 0.04 ^{b}
	2.0	85.00 ± 0.57 ^{c}	12.20 ± 0.16 ^{c}	12.20 ± 0.02 ^{b}
	2.5	80.66 ± 1.85 ^{e}	12.06 ± 0.24 ^{c}	10.47 ± 0.08 ^{d}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine; IBA = Indole-3-butyric acid

Quantification of Photosynthetic Pigments

Overall, 97% of the plants (derived from *m*T) and 84% (derived from BA) that survived the acclimatization process were used to quantify photosynthetic pigments. During the acclimatization period, the concentrations of pigments were lower in the first week; later, they increased during the third and fourth weeks (Figure 2). When comparing the photosynthetic pigments (chlorophyll a, b and carotenoids) in the acclimatized plantlets after 4 weeks, there was a significant increase in the contents of photosynthetic pigments (chlorophyll a (9.2%), b (11.3%) and carotenoids (29.1%)) in *m*T-derived *in*

vitro regenerated plants (chlorophyll a, 178 µg/g; chlorophyll b, 98 µg/g; and carotenoid, 93 µg/g of FW) compared with BA-treated plants (chlorophyll a, 163 µg/g; chlorophyll b, 88 µg/g; and carotenoid, 72 µg/g of FW). The concentrations of all three assayed photosynthetic pigments increased and reached their maximum concentration in the fourth week of acclimatization (Figure 2). Similarly, Ahmad et al. (2018) reported that the carotenoid and chlorophyll concentrations increased during acclimatization and reached maximum during the fourth week in *Decalepis salicifolia*. This assessment strongly recommended that a stable increase in photosynthetic pigments is directly related to stress adaptation and proper physiological functions of acclimatized plantlets.

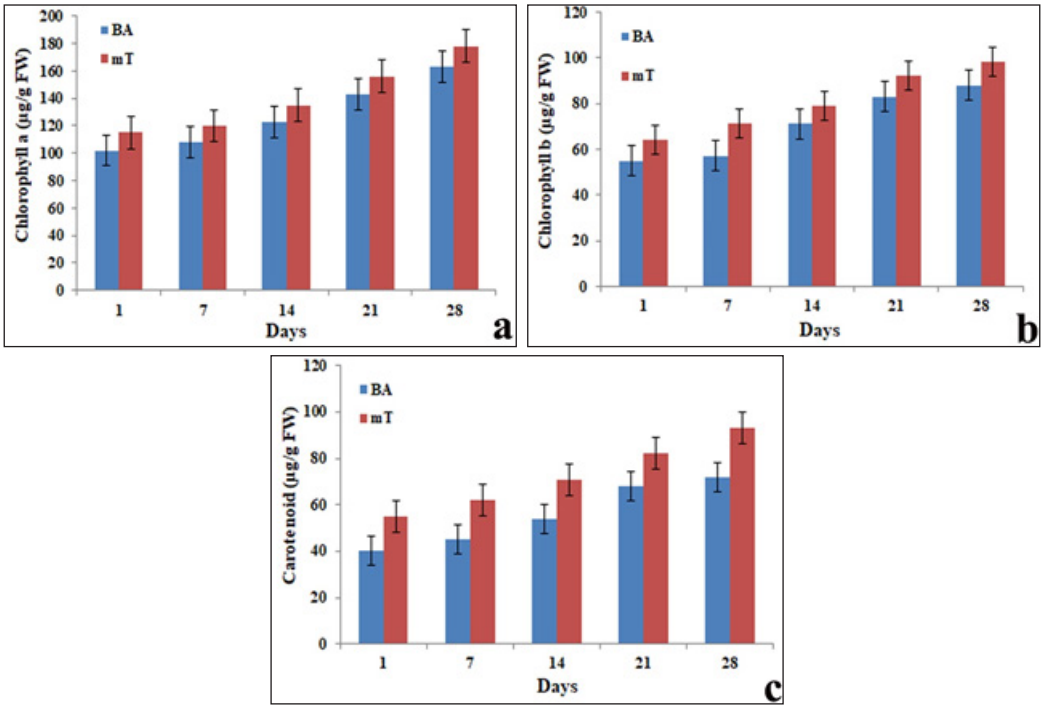

Figure 2. Effect of *m*T on photosynthetic pigments of *C. lanatus* (Arka Manik). a) Estimation of photosynthetic pigment (Chlorophyll a), b). Estimation of photosynthetic pigment (Chlorophyll b), c) Estimation of photosynthetic pigment (Carotenoid)

Interestingly, the carotenoid pigment level was drastically improved to 30% (93 µg/g of FW) in *m*T–derived plants compared to BA-treated plants (72 µg/g of FW) after four weeks of acclimatization. In accordance with our results, Jeon et al. (2005) confirmed that the carotenoids play a pivotal role in defending the photosynthetic apparatus from photo-oxidative injuries, and their elevation during acclimation was a result of the plants reacting to the stresses of acclimation, resulting in high survival of *in vitro* regenerated plantlets of *L. speciose*. In this study, the higher photosynthetic pigment accumulation in

the acclimatized plantlets implies that the *in vitro*-regenerated plantlets were successfully acclimatized to ensure proper growth. Our results are in accordance with a few studies where enhancement in chlorophyll and carotenoid content usually implies an increased rate of photosynthesis (Mahanta et al., 2023; Osorio et al., 2013). *Meta*-topolin regulates stress-associated problems and increases photosynthetic pigments, rooting, and acclimatization efficiency (Ahmad & Anis, 2019). Furthermore, they stated that *m*T lowers chlorophyllase activity and protects the photosynthetic pigments from environmental stresses during the acclimatization of plantlets.

Evaluation of Genetic Fidelity

The genetic fidelity of the *in vitro* regenerated watermelon plants with that of the mother plant was assessed by RAPD and SCoT markers. A monomorphic banding profile was obtained in all 10 RAPD and 15 SCoT primers. A total of 49 scorable and reproducible distinct bands of DNA were generated from 10 RAPD primers in the size range of 200 to 1800 base pairs (bp) (Table 4). A total of 49 scorable bands were obtained from all the RAPD primers with a maximum of 8 scorable bands per primer per sample observed in OPA1 primers (Figure 3a) with sizes ranging from 300-1800 bp wherein a minimum number of 2 bands were observed in OPA6 primer with size ranging from 400–1400 bp.

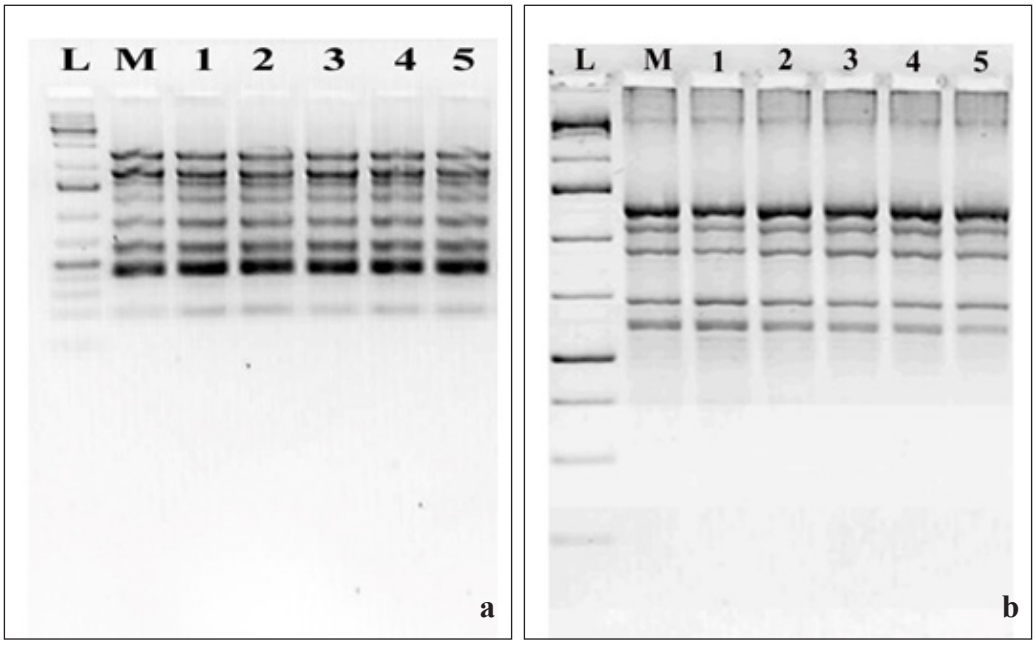

Figure 3. Genetic fidelity analysis of *in vitro* regenerated plants of watermelon. a) RAPD primer (OPA 01). Lane L-1 kb ladder, Lane M-Mother plant, lanes 1–5 Regenerated plants, b) SCoT primer (S16). Lane M-Mother plant, lanes 1–5 Regenerated plants. Lane L-1 kb ladder

Table 4
List of RAPD markers used for genetic fidelity analysis of regenerated watermelon

No.	Primer Name	Primer sequence (5′ – 3′)	Number of scorable monomorphic bands/primer	Size range of bands (bp)
1	OPA1	CAGGCCCTTC	8	300 - 1800
2	OPA2	TGCCGACCTG	5	300 - 1800
3	OPA6	GGTCCCTGAC	2	400 -1400
4	OPA7	GAAACGGGTG	5	500 - 1800
5	OPA8	GTGACGTAGG	5	200 - 1600
6	OPA11	CAATCGCCGT	6	400 - 900
7	OPA13	CAGCACCCAC	5	300 - 1200
8	OPA14	CTCGTGCTGG	4	200 - 1000
9	OPD13	GGGGTGACGA	4	400 -1200
10	OPD16	AGGGCGTAAG	5	200 -800
		Total	49	200 -1800

A total of 40 scorable and reproducible distinct bands of DNA were generated from 15 SCoT primers in the range of 300 to 2000 bp (Table 5). Among all the SCoT primers, the maximum number of 5 scorable bands were observed in S16 primers (Figure 3b) with sizes ranging from 600 to 1400 bp, while the minimum number of scorable bands was observed in S10, S17 and S26 primers with a molecular size range of 1700 bp, 1200bp and 1500 bp, respectively.

Table 5
List of SCoT markers used for genetic fidelity analysis of regenerated watermelon

No.	Primer name	Primer sequence (5′ –3′)	Number of monomorphic bands/primer	Size of bands (bp)
1	S1	CAACAATGGCTACCACCA	2	1200-1600
2	S2	CAACAATGGCTACCACCC	2	800-1000
3	S3	CAACAATGGCTACCACCG	4	500-2000
4	S4	CAACAATGGCTACCACCT	4	500-1500
5	S5	CAACAATGGCTACCACGA	3	300-1700
6	S6	CAACAATGGCTACCACGC	3	1000-1500
7	S7	CAACAATGGCTACCACGG	2	600-1500
8	S10	CAACAATGGCTACCAGCC	1	1700
9	S11	AAGCAATGGCTACCACCA	4	500-1200
10	S12	ACGACATGGCGACCAACG	2	300-1500
11	S16	ACCATGGCTACCACCGAC	5	600-1400
12	S17	ACCATGGCTACCACCGAG	1	1200
13	S25	ACCATGGCTACCACCGGG	2	1000-1600
14	S26	ACCATGGCTACCACCGTC	1	1500
15	S32	CCATGGCTACCACCGCAC	4	400-1400
		Total	40	300-2000

The banding pattern of all the 25 primers (10 RAPD and 15 SCoT primers) of *in vitro*-regenerated and subsequently acclimatized plantlets with the mother plant showed 100% monomorphism without any genetic variations. The DNA banding patterns of the RAPD and SCoT primers clearly revealed no discernible genetic variations, mutations, or polymorphisms among the regenerated watermelon plants. In many research reports, the use of the RAPD and SCoT marker method for evaluating the genetic fidelity of *in vitro*-regenerated plantlets has been effective (Ajithan et al., 2020; Bisht et al., 2024; Elayaraja et al., 2019; Joshi et al., 2023; Sathish et al., 2022; Vasudevan et al., 2017). Due to its affordability, simplicity, and repeatability, the RAPD and SCoT marker system combination is deemed effective.

CONCLUSION

This study highlights the positive effect of *m*T over the commonly used cytokinin (BA) in attaining double the number of multiple shoots from the cotyledonary node explants of watermelon. The potential of *m*T in improving shoot multiplication and subsequent rooting and its favorable impact on the acclimatization of regenerated plants were investigated for the first time in watermelon. With the aid of molecular markers, RAPD and SCoT, this study also confirmed that the plantlets produced using *meta*-topolin (1.5 mg/L *m*T followed by 1 mg/L IBA for rooting) was true to type. During acclimatization, the plantlets had higher success (97%) with increased photosynthetic pigment concentration (Chlorophyll a, b, and carotenoids) that supported vigorous plant growth and development.

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